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Cell-Specific Expression of the Promoters of Two Nonlegume Hemoglobin Genes in a Transgenic Legume, *Lotus corniculatus*

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The promoters of the hemoglobin genes from the nitrogen-fixing tree *Parasponia andersonii* and the related nonnitrogen-fixing *Trema tomentosa* both confer β-glucuronidase reporter gene expression to the central zone of the nodules of a transgenic legume, *Lotus corniculatus*. β-Glucuronidase expression was high in the uninfected interstitial cells and parenchyma of the surrounding boundary layer and was low in the *Rhizobium*-infected cells. This contrasts with the expression of both the *P. andersonii* hemoglobin protein in *P. andersonii* nodules and the endogenous *Lotus* leghemoglobins that are expressed in the infected cells at very high levels. The expression pattern of the *P. andersonii* and *T. tomentosa* hemoglobin promoters in *L. corniculatus* resembles that of a nonsymbiotic hemoglobin gene from *Casuarina glauca*, which was introduced into this legume, and suggests that only the nonsymbiotic functions of the *P. andersonii* promoter are being recognized. Deletion of the distal segments of both the *P. andersonii* and *T. tomentosa* promoters identified regions important for the control of their tissue-specific and temporal activity in *Lotus*. Potential regulatory elements, which enhance nodule expression and suppress nonnodule expression, were also identified and localized to a distal promoter segment. A proximal AAGAG motif is present in the *P. andersonii*, *T. tomentosa*, and nonsymbiotic *Casuarina* hemoglobin genes. Mutation of this motif in the *P. andersonii* promoter resulted in a significant reduction in both the nodule and root expression levels in *L. corniculatus*. Some of the regulatory motifs characterized are similar to, but different from, the nodulin motifs of the leghemoglobins.

Hemoglobin is present in the nitrogen-fixing nodules of both legumes and nonlegumes, in which it facilitates the diffusion of oxygen to the nitrogen-fixing symbiont (*Rhizobium* or *Frankia*) to maintain the free oxygen tension at a sufficiently low level to avoid inhibiting the oxygen-sensitive bacterial nitrogenase enzyme (reviewed by Appleby, 1984). Hemoglobin has also been identified in nonnodule tissues of nodulating nonlegume plants and in other nonnodulating plants (Bogusz et al., 1988). *Casuarina glauca* is a nodulating nonlegume species, which has both symbiotic (nodule-specific) and nonsymbiotic hemoglobins (expressed in nonnitrogen-fixing tissues) (Fleming et al., 1987; Christensen et al., 1991; Jacobsen-Lyon et al., 1995). Nonsymbiotic hemoglobin genes have recently been identified in monocotyledonous plants such as barley, wheat, maize, and rice (Taylor et al., 1993, 1994; Andersson et al., 1996). The function of these plant hemoglobins in nonsymbiotic tissues is not clear; they may be associated with the transport of oxygen or, as suggested by Appleby et al. (1988, 1990), they may act as oxygen sensors in the signal transduction pathway for activation of anaerobic genes.

Legumes typically contain a number of hemoglobin (or leghemoglobin) genes, which are expressed at high levels only in nodules (Marcker et al., 1984). The promoter regions of the soybean *lbc3* and *lba* genes, and of the *Sesbania rostrata glb3* gene, have each been linked to a reporter gene and introduced into the legume *Lotus corniculatus* (Stougaard et al., 1987; Szabados et al., 1990; She et al., 1993). These legume promoters direct high levels of gene expression in *Rhizobium*-infected cells located in the central region of nodules formed on transgenic *L. corniculatus* roots (Lauridsen et al., 1993; She et al., 1993). These are the same cells in which the endogenous *L. corniculatus* leghemoglobins are expressed. This indicates that the tissue- or organ-specific signals required for nodule expression are functionally conserved between legume genera.

Promoter regions important for high-level nodule expression, containing enhancer and repressor elements and an OSE, have been identified in the soybean *lbc3* gene (Stougaard et al., 1987, 1990). A promoter region containing the OSE conferred nodule specificity when linked to a minimal region of the CaMV 35S promoter (Stougaard et al., 1987). A NICE has been identified within an analogous region of the *S. rostrata glb3* promoter (Szczyglowski et al., 1993) and was able to direct enhanced nodule expression when fused to a minimal NOS promoter. Both OSE and NICE contain the highly conserved nodulin consensus motifs AAAGAT and CTCTT found in all leghemoglobin promoters and in the *C. glauca* symbiotic hemoglobin promoter

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Abbreviations: CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyltransferase; NICE, nodule-infected cell element; NOS, nopaline synthase; OSE, organ-specific element.

(Fig. 1), as well as many other nodule-expressed (nodulin) genes (Sandal et al., 1987; Metz et al., 1988). These motifs are generally separated by 6 to 7 bp.

The Australasian tree *Parasponia andersonii*, a member of the elm family, is the only nonlegume known to form nitrogen-fixing nodules in symbiosis with *Rhizobium*. It has a single hemoglobin gene expressed at high levels in nodules and at much lower levels in roots (Landsmann et al., 1986; Bogusz et al., 1988). The closely related species *Trema tomentosa* does not form nodules but has a similar hemoglobin gene expressed in root cells (Bogusz et al., 1988). The *P. andersonii* and *T. tomentosa* hemoglobin promoters have been shown to direct high levels of GUS expression in the central, infected zone of the transgenic nodules of *L. corniculatus* (Bogusz et al., 1990). Although this suggested that the signals responsible for nodule expression may be the same in the legume and nonlegume nitrogen-fixing symbioses involving *Rhizobium*, the cell-type specificity of the expression within the transgenic nodules of these nonlegume promoters was not determined. The two promoters both contain the AAAGAT nodulin motif. The *T. tomentosa* promoter contains a variant AAGGAG and an exact AAAGAT further upstream (Fig. 2). The pyrimidine-rich sequence CTCTT, normally 5 bp downstream from the AAAGAT motif in all the leghemoglobins (except for the soybean pseudogene *lbps1*), is not present in the *T. tomentosa* and *P. andersonii* promoters (Figs. 1 and 2). However, further downstream of the AAAGAT motif is the sequence AAGAG (the complement of the CTCTT nodulin motif), which is present in all of the nonsymbiotic hemoglobin promoters so far characterized (Fig. 1). This arrangement of the spaced AAAGAT and AAGAG motifs (16–18 bp apart) does not occur in any of the described symbiotic hemoglobin promoters.

Because transformation systems do not exist for *P. andersonii* and *T. tomentosa*, we conducted an analysis of their hemoglobin promoters in transgenic *L. corniculatus* tissues. Our goals were to identify regions responsible for organ-specific expression, particularly those relating to differential root and nodule expression, and to compare the regulation of hemoglobin expression in legumes and nonlegumes. We demonstrate that, although the *P. andersonii* and *T. tomentosa* promoters direct expression in *L. corniculatus* nodules, the cell-type specificity within the nodule is not the same as that of the endogenous leghemoglobins. The role of two promoter motifs related to sequence motifs critical for leghemoglobin expression was investigated by site-directed mutagenesis, and using 5′ deletions, we have also shown the presence of tissue-specific and repressor elements in the two promoters.

MATERIALS AND METHODS

Plasmid Constructions

The *Parasponia andersonii* and *Trema tomentosa* hemoglobin promoters have previously been transcriptionally fused to the *Escherichia coli* GUS reporter gene (*uidA*) and to the NOS transcription termination and polyadenylation signals in the pGN100 expression cassette (Bogusz et al., 1990) by introducing a *Bam*HI restriction site adjacent to the hemoglobin translation start codons (Bogusz et al., 1990) (Fig. 2). The T-278 construct (*T. tomentosa* hemoglobin promoter deleted to −278 relative to the transcription start site) was made as an *Nco*I/*Bam*HI promoter fragment (Fig. 2) inserted in pGN100. The P-235 construct (*P. andersonii* hemoglobin promoter deleted to −235 relative to the transcription start site) was made as a *Bst*NI/*Bam*HI promoter fragment (Fig. 2) inserted in pGN100. Both the full-length and the T-278 and P-235 promoter-GUS constructs were used for generating unidirectional 5′ deletions (Nested Deletion Kit, Pharmacia). Deletion end points were con-

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-140  AGTTTTTGAAAAGAT-GATTGTCTCTTCACCATACCA---ATTGATCACCCTCC---TCCAACAAGCCAAGAG  -75  lbc3
-132  AATTTTTTAAAAGAT-CGTTGTTTCTTCTTCATCATGCTGATTGA-CACCCTCC-------ACAAGCCAAGAG  -69  lba
-127  GGATTTTGAAAAGAT-CATTGGCTCTTCGTCATGCCG---ATTGA-CACCCTCC-------ACAAGCCAAGAG  -67  lbc1
-129  AGATTTTGAAAAGATCATTTGGCTCTTCATCATGCCG---ATTGA-CACCCTCC-------ACAAGCCAAGAG  -67  lbc2
-150  AGTTTTTGAAAAGGT-CGTTGTGTCTTCATAATGCCG---ATTGAT-ACGCTCCACATTCAATAAGCCAAGAG  -83  lbps1

-201  AATTTTTAAAAGAT-TATTGTCTCTTAATAATGTCA---ATGGC-CACCCTTT-------AGGAGCCAAGAG -141  glb3
-201  AATTTTTAAAATGAT-TATTGTCTCTTAATAATGTCA---ATGGC-CACCCTTC-------AGGAGCCAAGAG -141  glb2
-147  AGTTTTTGAAAAGTT-TTTTGTCTCTTAATAACTACA---ATGGT-CACCTCC--------ACAAGCCATTAT  -88  Mtlb1
-151  AGTTTTTTAAATGAT-TATTGTCTCTTTAATAATGTCA--ACAGC-CATTTCC--------ACAAGCCAATAG  -91  Mtlb2
-206  AGTTTTTGAAAAGAT-TATTGTCTCTTTAATGTTGTCA--ATAGC-CATTTCC--------ACAAGCCAATAG -146  Ms1
-201  AGTTTTTTAAATGAT-TATTGTCTCTTTAATAACGTCA--ACGGC-CATTTCC--------ACAAGCCAATAG -141  Msplb2

-489  CTTCAATCCCAAGATGTCCTCTCTCTTATTGATATTTGAACAACAACAAAGATAAACAACCATTATCCCTACC -418  Cassym1
-323  CTTCAATCCCAAGATGTCCTCTCTCTTATTGATATTTGAACAACAACAAAGATAAACAACCTTTATCCTTACC -251  Cassym1
-207  AATTGACCCAAAGAA--ATGG-CTTTC---GACCCACGAAGAGCCGGAGCTATCCCTGTTACG-TGCGCTATA -142  Casnonsym

-108  AAAAAACCCAAAGAT--ATGG-CTCCCCAATACCCT-GAAGAGTTACACACGA----------TCCCCATTT  -51  Parasponia
-116  AAAAAACCCAAGGAG--ATGG-CTCTCCAGTACCCT-GAAGAGTTACATCTTATGATCTTA---TCCCCCATT  -51  Trema
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Figure 1. Alignment of the "nodulin box" motifs of leghemoglobin and hemoglobin gene promoters. Soybean *lbc3*, *lba*, *lbc1*, *lbc2*, and *lbps1* (Stougaard et al., 1987) are numbered from the transcription start site. *S. rostrata glb3* and *glb2* (Metz et al., 1988) are numbered from the ATG codon. *Medicago truncatula Mtlb1* and *Mtlb2* (Gallusci et al., 1991) are numbered from the transcription start site. *Medicago sativa Ms1* and *Msplb2* (Davidowitz et al., 1991) are numbered from the ATG codon. The *C. glauca Cassym1* sequence (Jacobsen-Lyon et al., 1995) is numbered from the ATG codon and includes the duplicated region containing the nodulin motifs. *C. glauca Casnonsym* (Christensen et al., 1991) is numbered from the ATG codon. *P. andersonii* hemoglobin (Landsmann et al., 1986) is numbered from the transcription start site. *T. tomentosa* hemoglobin (Bogusz et al., 1988) is numbered from the transcription start site and inferred from the alignment with the *Parasponia* gene. The conserved nodulin motifs AAAGAT and CTCTT, the purine-rich motif AAGAG found in the nonsymbiotic genes just downstream of the AAAGAT motif, and the similar motif found in many of the leghemoglobin genes are indicated in bold type.

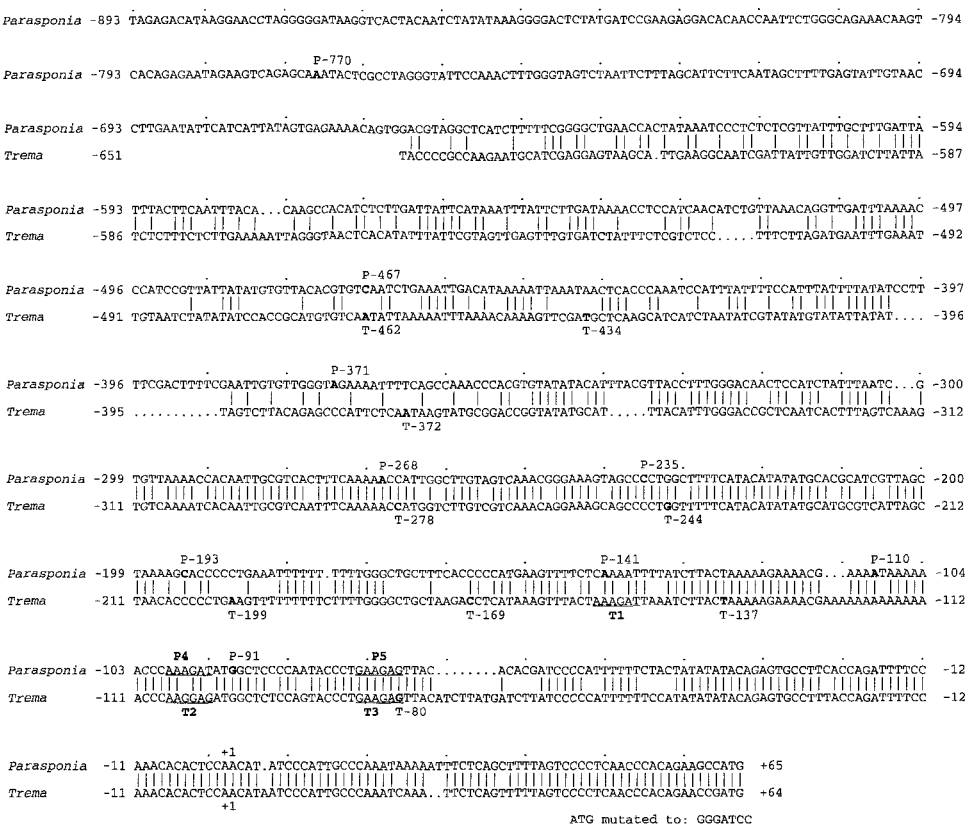

Figure 2. Alignment of the *P. andersonii* and *T. tomentosa* hemoglobin promoter sequences showing the end points of 5′ deleted promoter fragments used in this study. The first base of the deleted promoters is indicated in bold type, and the corresponding construct is designated above or below the sequence as described in the text. The putative nodulin consensus motifs altered by site-directed mutagenesis are underlined and labeled in bold as T1 to T3 and P4 and P5. Numbering is as indicated in Figure 1. +1 is the transcription start site determined for the *P. andersonii* gene. The region around the translation start of both promoters was mutated to GGATCC as indicated to allow fusion to the GUS reporter gene as described by Bogusz et al. (1990).

firmed by dideoxy sequencing using T7-sequencing reagents (Pharmacia). Individual deletions are referred to as either T-xxx or P-xxx, respectively, for *T. tomentosa* or *P. andersonii* promoters deleted to nucleotide −xxx relative to the transcription start. All promoter-GUS-NOS fragments were transferred as *Eco*RI/*Hin*dIII fragments to the co-integrative vector pIV10 (Ramlov et al., 1993). The extended *P. andersonii* promoter sequence has been submitted to the EMBL database under accession no. U27194. The sequence of the *T. tomentosa* hemoglobin promoter (Bogusz et al., 1988) has also been corrected at several positions.

The soybean *lbc3*-GUS-NOS construct in pIV10 (Lauridsen et al., 1993) was used as a control to indicate the expression of a typical leghemoglobin gene in transgenic *Lotus corniculatus* nodules and roots.

Site-Directed Mutagenesis

The following synthetic oligonucleotides were used to introduce mutations into the putative nodulin boxes identified in Figure 2: T1, (−168) 5′-CTCATAAAGTTTACTGGTACCTAAATCTTACTAAAAA; T2, (−122) 5′-AAAAAAAAAAAACCCGGTACCATGGCTCTCCAG; T3, (−95) 5′-CTCCAGTACCCTTCTAGATTACATCTTATGATC; P4, (−114) 5′-GAAAATAAAAAACCCGGTACCATGGCTCCCCA; and P5, (−87) 5′-CCCCAATACCCTTCTAGATTACACACGATCC.

The full-length promoters in pUC118 (*P. andersonii*) or pUC119 (*T. tomentosa*) were used as a template. Single-stranded plasmid DNA isolated from a *dut ung* strain of *E. coli* was annealed with the appropriate oligonucleotide. Replication of the plasmids was completed with deoxynucleotides T4 DNA polymerase and T4 DNA ligase, and they were then introduced into *E. coli* strain DH5α. Putative mutants were screened by colony hybridization using the end-labeled oligonucleotide probes. The presence of the appropriate mutation was confirmed by dideoxy sequencing. To create promoters with mutations in two promoter motifs, plasmids with mutated "AAGAG" boxes (T3 and P5) were used as a template for the introduction of a second mutation.

The mutated promoters were introduced as *Bam*HI (*P. andersonii*) or *Bam*HI/*Eco*RI (*T. tomentosa*) fragments into the pIV20 co-integrative vector. pIV20 was constructed by transferring the *Eco*RI/*Hin*dIII GUS-NOS cassette from

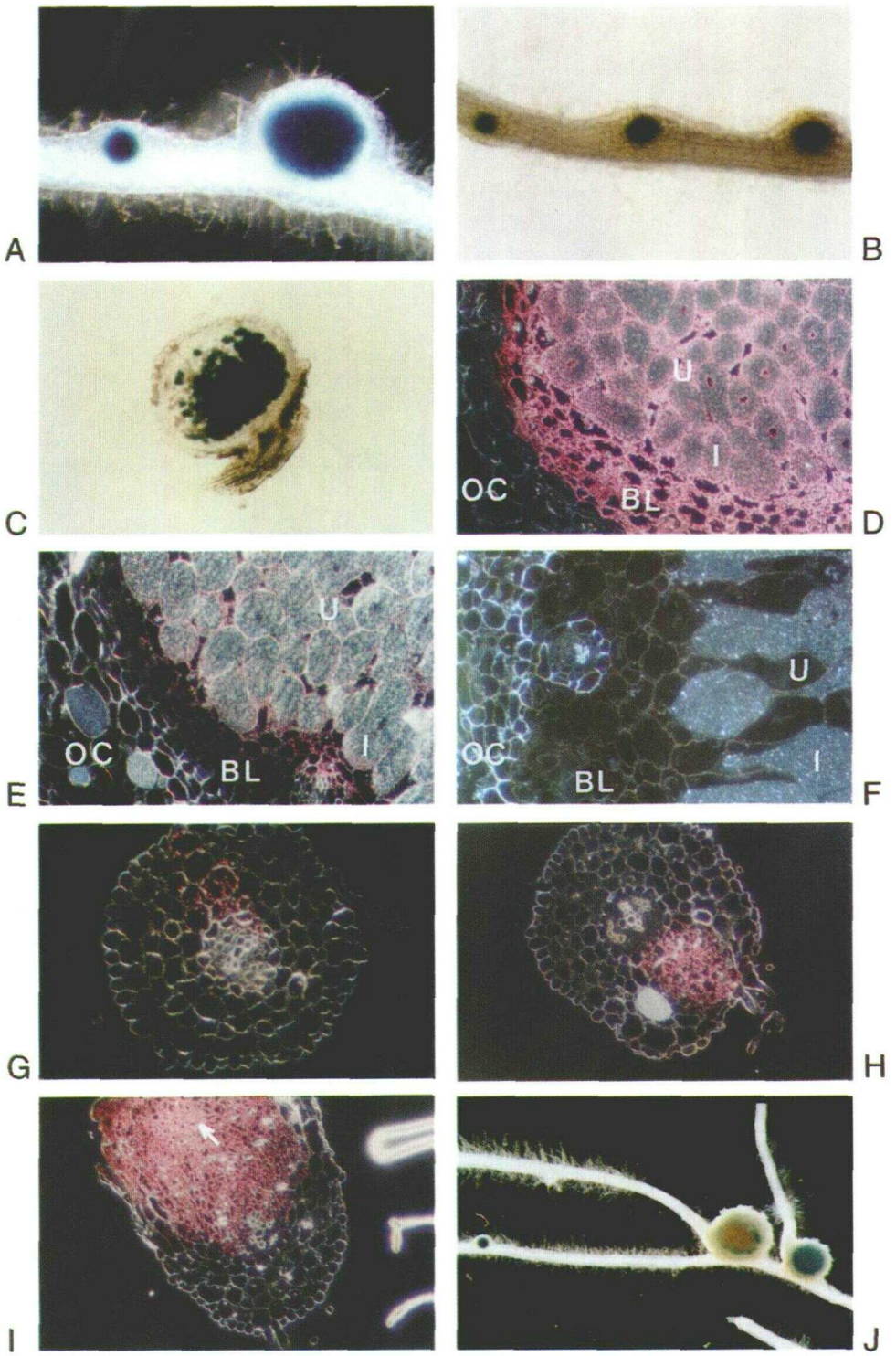

Figure 3. (Figure continues on facing page.)

pGN100 to pIV10 to yield a plasmid similar to pIV21 (Ramlov et al., 1993) but with the multiple cloning site in the reverse orientation. Correct insertion was confirmed by restriction digest analysis. Dideoxy sequencing using a primer to the GUS-coding region confirmed correct fusion at the promoter-GUS junction and the presence of the correct mutations.

Plant Transformations

Co-integrative vector constructs were introduced into *Agrobacterium rhizogenes* strain AR14 (Hansen et al., 1989) by biparental mating. The AR14 strain carries a CaMV 35S-CAT-E9 marker gene, as well as pBR322 sequences, allowing integration of the pBR322-derived pIV vector se-

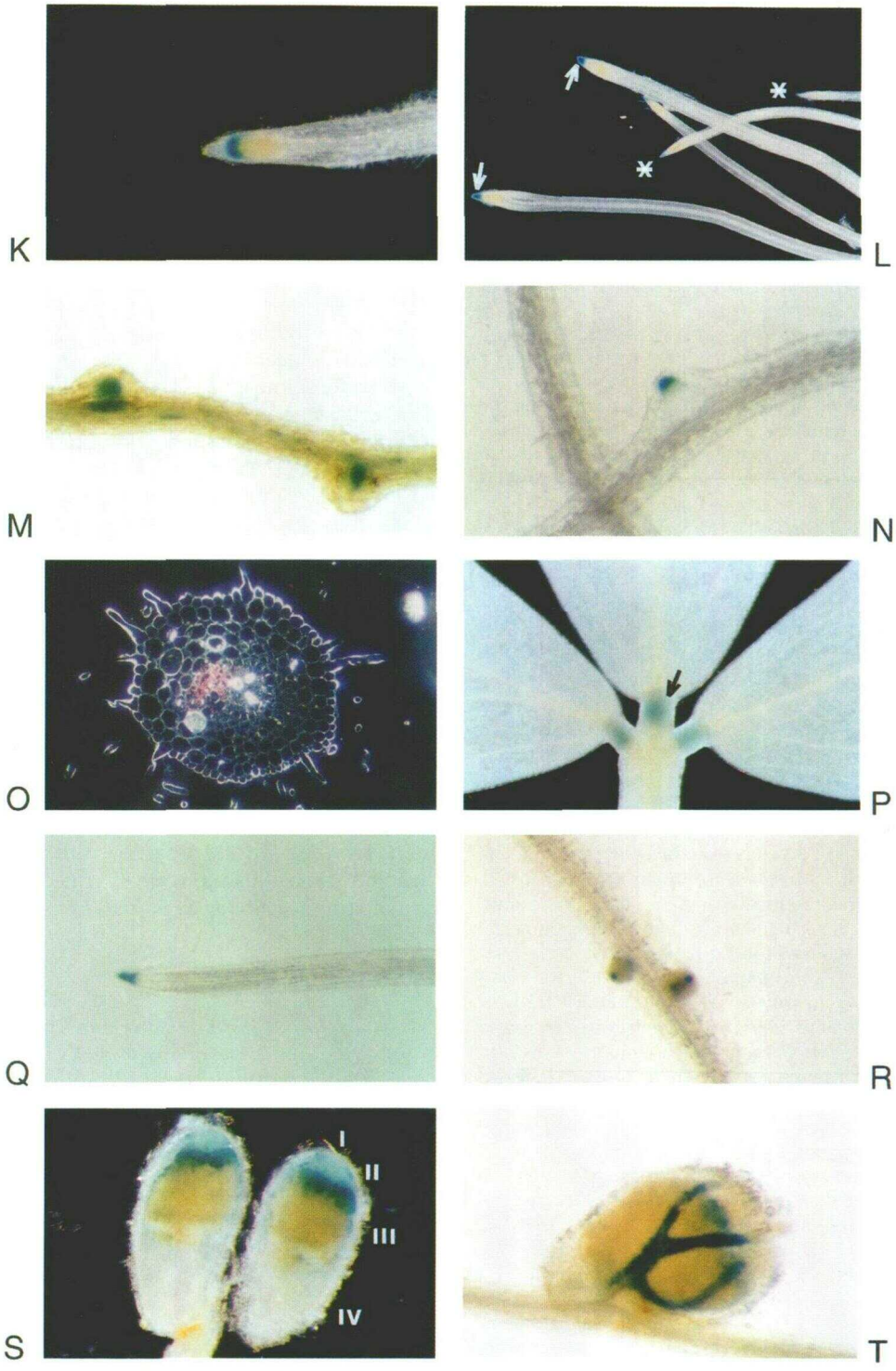

Figure 3. (Continued from previous page.)

Figure 3. Histochemical localization of GUS activity directed by *P. andersonii* and *T. tomentosa* hemoglobin promoter GUS fusions in transgenic *L. corniculatus* and *T. repens*. A, Full-length *P. andersonii* hemoglobin promoter expressing GUS (P-GUS) within the infected zone of transgenic *L. corniculatus* nodules at two developmental stages (×29). B, Full-length *T. tomentosa* GUS (T-GUS) showing expression in *L. corniculatus* nodules of different developmental stages (×29). C, Hand-cut section of a *L. corniculatus* nodule transformed with the lbc3GUS construct showing GUS staining in the infected cells of the central region (×33). D, P-GUS expression in part of a sectioned, mature *L. corniculatus* nodule photographed under dark-field (the crystals of the GUS product appear pink; ×115). D to F, The bluish appearance of the large infected cells is a characteristic of the cells and is not indicative of GUS activity. In this section, expression can be seen in the partially

ries between the Ri-DNA border sequences (Ramlov et al., 1993). Integration was confirmed by Southern blot hybridization analysis of total DNA using probes for both the hemoglobin promoter and the GUS-coding region.

L. corniculatus (cv Rodeo) and *Trifolium repens* (cv Milkanova) plants with transformed hairy roots on untransformed shoots were generated as described by Hansen et al. (1989) and were used for all the analyses of nodule and root expression described in this study. Transgenic nodules were induced after inoculation with a 2-d-old culture of either *Rhizobium loti* NZP2037 or *R. leguminosarum* bv *trifolii* ANU843, and the plants were analyzed after 4 to 6 weeks. In selected cases hairy roots were removed and placed in tissue culture and whole transformed *L. corniculatus* plants were produced as described by Petit et al. (1987). These plants were screened for the linked CAT marker gene and confirmed transgenics were transferred to soil in a containment greenhouse to examine transgene expression in above-ground tissues.

Figure 3. (Legend continued from p. 49.)
sclerified parenchyma of the boundary layer (BL) surrounding the central infected zone, in the uninfected interstitial cells (U), as well as at low levels in the large infected cells (I). No expression was seen in the outer cortex (OC) of the nodule. E, T-GUS in sectioned *L. corniculatus* nodule, showing a similar, but weaker, pattern of expression to P-GUS in D (×115). Staining in the boundary layer (BL) was very faint. F, A control, nontransformed *L. corniculatus* nodule stained for GUS was sectioned and examined under dark-field. No pink GUS product is evident, although some starch grains show up as white spots in the infected cells (×115). G, P-GUS in a transverse section of a *L. corniculatus* root (×172), showing GUS expression in a nodule primordium as a few stained cells extending from the outer cortex to the pericycle prior to any evidence of cell division or perturbation of the root surface. H, P-GUS expression in transverse section of a *L. corniculatus* root (×111), showing GUS expression in a nodule primordium at a later developmental stage when significant cell division has occurred. Expression extends from the outer cortex to the pericycle. I, P-GUS expression in a sectioned *L. corniculatus* root showing a developing nodule as it erupts through the root cortex (×107). Staining is intense in the nodule meristem (arrow). J, P-770 transformed roots showing the differential expression of GUS in *L. corniculatus* nodules of different developmental stages (×7). K, T-278 transformed roots expressing GUS in the meristem initial zone of a *L. corniculatus* root tip (×45). L, P-467 transformed roots showing the differential expression of GUS in *L. corniculatus* root meristem initial cells (×6). Staining was strongest in the initial cells of the branches from the primary root most distal to the stem (arrow). Weaker staining was seen in the root tips (asterisk) from branches more proximal to the stem. M, P-371 transformed *L. corniculatus* root, showing the GUS expression in a rounded nodule primordium (left) and elongate root primordium (right) (×15). N, P-371 transformed roots expressing GUS in the newly formed cap and meristem initial cells of an emerging lateral root of *L. corniculatus* (×49). O, Transverse root section of T-278 transformed *L. corniculatus* (×115). GUS staining is confined to the cells of the pericycle, endodermis, and inner cortex, indicating that this is a lateral root primordium (×115). The pattern of cell division and GUS staining differs from that of P-GUS in the developing nodule primordium (G and H). P, P-467 directed GUS expression in the secondary pulvini (arrow) of a transgenic *L. corniculatus* plant (×29). Q, T-274 directed GUS expression in the meristem initials of a transgenic white clover root (×41). R, P-GUS expression in very young white clover nodules prior to the onset of nitrogen fixation (×49). S, P-GUS white clover transformant showing expression in a bisected mature nodule in the early symbiotic zone (interzone II/III) (×49). Low expression was sometimes observed in the nitrogen-fixing zone (III), little was observed in the meristematic zone (I), and none was observed in the senescent zone (IV). T, P-GUS expression in the the early symbiotic zone and nodule vasculature of a mature white clover nodule (×49).

GUS Histochemical Assay

Excised roots and nodules were stained using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Progen Industries, Brisbane, Queensland, Australia) in a buffer containing 10 mM sodium phosphate, pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 10 mM EDTA (Jefferson, 1987). Tissue was briefly vacuum-infiltrated and incubated at 37°C in the dark for 12 to 24 h. Large nodules were incised prior to staining to ensure penetration of the substrate. Above-ground tissue was stained in the same solution supplemented with 0.01% Tween 60 and then cleared of pigment by washing in a graded ethanol series, followed by rehydration in 10 mM sodium phosphate, pH 7.0. Stained tissue was photographed using either bright- or dark-field optics.

For cellular localization of GUS histochemical activity, stained tissue was fixed (3% glutaraldehyde in 25 mM sodium phosphate, pH 7.0) overnight and dehydrated through a graded ethanol series. Tissue was infiltrated in London Resin White (London Resin Co., London, UK) for several days and polymerized at 60°C under nitrogen for 2 h. Two- to 3-μm-thick sections (ultramicrotome, Reichert-Jung, Vienna, Austria) were mounted in immersion oil and photographed under dark-field optics.

GUS Fluorometric Assay

Quantitative GUS assays were performed on excised nodules or roots essentially as described by Jefferson (1987) using 4-methylumbelliferyl β-D-glucuronide (Sigma) as a substrate. The rate of production of 4-methylumbelliferone was measured using a Fluoroskan II microplate fluorometer (Labsystems, Research Triangle Park, NC) and expressed as fluorescence units min^{-1} mg^{-1} protein. Protein concentration was determined by the method of Bradford (1976) and measured using a Multiskan Plus microplate reader (Labsystems).

RESULTS

Cell Specificity of the *P. andersonii* and *T. tomentosa* Promoters in Transgenic *L. corniculatus* Nodules Differs from the Endogenous Hemoglobin Genes

The *P. andersonii* hemoglobin promoter linked to a GUS reporter gene directs GUS expression in the cells of the bacteroid-containing region of transgenic *L. corniculatus* nodules (Fig. 3A, cf. Bogusz et al., 1990). The use of thin sections and dark-field microscopy on stained tissues has allowed us to examine in more detail the cell-type speci-

ficity within this region and, contrary to expectations, we found only low levels of GUS staining in the bacteroid-containing cells of the mature nodule and a larger amount of intense staining in the uninfected, interstitial cells (Fig. 3D). Activity was also detected in the specialized parenchyma of the boundary layer (inner cortex) surrounding the infected region (Fig. 3D) and in the parenchyma around the nodule vascular trace (not shown). No GUS staining was observed under either bright-field or dark-field illumination when nontransgenic or transgenic nodules containing a promoterless GUS gene were stained under identical conditions (Fig. 3F). This expression pattern for the nonlegume hemoglobin GUS constructs contrasts with that of both the endogenous *L. corniculatus* leghemoglobins, the soybean *lbc3* (Fig. 3C) and *lba* promoters (Lauridsen et al., 1993; She et al., 1993), and the *Casuarina glauca* symbiotic hemoglobin promoter (Jacobsen-Lyon et al., 1995) in transgenic *L. corniculatus*. These genes are expressed almost exclusively in the infected cells of the central zone.

The staining of different-sized nodules indicated that the *P. andersonii* promoter was active at all stages of nodule development, including very small white nodules not yet capable of nitrogen fixation (Fig. 3A; Table I). During the initial stages of nodule morphogenesis, GUS staining was localized to relatively few cells extending from the outer cortex to the pericycle (Fig. 3, G and H). At slightly later stages of nodule development GUS expression appeared to occur throughout the entire nodule cell mass, with intense staining in the nodule meristem (Fig. 3I). Occasionally, GUS activity was detected outside nodules in the root vasculature of the region of nodule attachment (not shown).

In transgenic *L. corniculatus* plants that were regenerated from root cultures transformed with the P-GUS construct, no staining was detected in leaves, shoots, stems, flowers, or roots (not shown). Although some GUS activity was seen in the pollen of all regenerated plants, this was also observed in pollen from regenerated control plants that did not contain a GUS gene, indicating that it was an artifact of the staining procedure. There have been other reports of endogenous GUS "activity" in pollen (Plegt and Bino, 1989; Kosugi et al., 1990).

Although GUS staining was not observed in the roots of any plants transformed with the full-length *P. andersonii* promoter GUS construct (Table I), Bogusz et al. (1990) reported low levels of staining in the root vasculature. The only difference between the two experiments is in the strain of *A. rhizogenes* used for the root transformations. Quantitative differences in hormone levels resulting from differences in *Rol* gene expression from the Ri-DNA in the

Table I. *Staining results for P. andersonii and T. tomentosa hemoglobin promoter-GUS constructs in transgenic hairy roots and regenerated plants of L. corniculatus*

P-xxx and T-xxx represent hairy roots or plants transformed with various deletion constructs of the *P. andersonii* or *T. tomentosa* promoters, respectively, deleted to nucleotide -xxx relative to the transcription start. All numbers represent independent transformants. The second column indicates the fraction of independent transformants showing some form of GUS activity out of the total number of independent transformants tested for each construct. The following columns indicate the number of independent transformants showing expression in a particular tissue. Large nodules were pink and 1.5 to 3.0 mm in diameter, whereas small nodules were white and less than 1.5 mm in diameter. For the pulvini expression, only the presence (+) or absence (−) of GUS expression in whole regenerated plants of selected transgenic lines is indicated. The soybean leghemoglobin *lbc3*GUS construct (Lauridsen et al., 1993) was used as a positive control.

Construct	No. of Transformants Expressing GUS Activity in Particular Tissues					
	Fraction expressing GUS	Large nodules	Small nodules	Distal root caps or root meristem initials	Emerging lateral root meristem initials	Pulvini
PGUS (P-893)	33/44	33	33	0	0	−
P-770	6/14	6	6	3	1	nt[a]
P-467	5/12	2	5	4	0	+
P-371	10/17	2	8	8	2	+
P-268	2/10	0	1	1	0	nt
P-235	11/33	0	11	0	0	nt
P-193	4/11	0	04	0	0	nt
P-141	3/15	0	3	0	0	nt
P-110	5/15	0	5	0	0	nt
P-91	0/10	0	0	0	0	nt
P-58	0/13	0	0	0	0	nt
TGUS (T-651)	13/48	13	13	2	0	−
T-462	5/13	3	3	3	3	+
T-434	2/3	0	2	2	1	nt
T-372	6/12	3	6	5	2	+
T-278	10/38	1	7	8	3	nt
T-244	5/21	0	2	3	0	nt
T-199	2/12	0	2	0	0	nt
T-169	0/14	0	0	0	0	nt
T-137	0/7	0	0	0	0	nt
T-80	0/6	0	0	0	0	nt
*lbc3*GUS	25/47	25	0	0	0	−

[a] nt, Whole transgenic plants were not generated and hence were not tested for GUS expression in pulvini.

transformed roots might have an impact on hemoglobin promoter function in the two different experimental systems. We observed that reporter gene expression levels were generally lower, although nodulation efficiencies were higher, with the AR14 strain used in this study compared with the A4 strain used by Bogusz et al. (1990). Hormonal influences on the *P. andersonii* or *T. tomentosa* promoters, to our knowledge, have not been previously studied; they may explain a number of the expression patterns we observed in transgenic *L. corniculatus* tissues.

The *T. tomentosa* promoter showed a similar pattern of nodule expression to the *P. andersonii* promoter (Fig. 3, B and E), although at much lower levels and perhaps with less pronounced differences in expression between the uninfected and infected cells. As with the *P. andersonii* construct, expression was also observed in the parenchyma cells of the boundary layer surrounding the central region, but the staining was very faint (Fig. 3E). GUS activity was occasionally seen in the root caps or root meristem initial cells (Table I) in roots containing the TGUS (T-651) construct. Control nodules from untransformed plants or plants transformed with a promoterless GUS gene construct, when stained, sectioned, and examined under dark-field conditions, contained few if any of the pink crystals indicative of GUS activity (Fig. 3F). The staining in TGUS nodules is consistent with previous (Bogusz et al., 1990) quantitative determinations of GUS activity in transgenic *Lotus* nodules that indicated that the *T. tomentosa* hemoglobin promoter directed significant activity above background.

A Distal Promoter Element Is Required for High-Level, Nodule-Specific Expression

Sequential 5′ deletions of the two promoters were introduced into *L. corniculatus* hairy roots using *A. rhizogenes* and the GUS reporter gene assayed in roots and nodules after nodulation by an appropriate *Rhizobium* strain. Although only semiquantitative, histochemical staining was used to follow GUS activity because reporter gene expression was detected in only a small proportion of the plants analyzed (Table I) and then not in every nodule on each transgenic root. This was despite the presence in most plants analyzed of both the characteristic "hairy root" transformation phenotype and CAT activity driven by the linked CaMV 35S-CAT marker gene. It is assumed that in some transformants the reporter gene construct may have been silenced, perhaps as a result of its site of insertion or through the mechanism of co-suppression by multiple Ri-DNA inserts. Sufficient expressing plants with stained nodules were obtained to characterize each of the promoter deletion constructs (Table I).

Deletion of the *P. andersonii* promoter from nucleotides −893 to −770, relative to the transcription initiation site (P-770), visibly reduced GUS staining in large pink nodules, but intense staining was still observed in small white nodules (Fig. 3J). This deletion defined a region between nucleotides −893 and −770 in the *P. andersonii* promoter required for high-level expression in mature nodules. Sequential deletion of the *P. andersonii* promoter to nucleotides −467 and −371 (P-467 and P-371) and the *T. tomentosa* promoter to nucleotides −462 and −372 (T-462 and T-372) resulted in further visible reductions in reporter gene staining in the nodules. Staining was low or absent in large mature nodules, whereas significant activity was observed in the young nodules (Table I). However, promoter deletions below nucleotides −371 or −278 (constructs P-371 or T-278, respectively) were only able to direct GUS activity in young white nodules, and no staining was detectable in any of the mature nodules assayed.

P. andersonii promoter deletions down to nucleotide −110 (P-110) were still able to direct significant levels of GUS expression in young nodules, but deletion of the *P. andersonii* promoter to nucleotides −91 and −58 eliminated histochemically detectable GUS expression in nodules of any size. In contrast, *T. tomentosa* promoter deletions of greater length than that in the P-110 construct (e.g. T-137 or T-169) were not able to produce histochemically detectable levels of GUS in young nodules (Table I), suggesting that the *T. tomentosa* hemoglobin promoter is generally weaker than the equivalent length *P. andersonii* promoter in transgenic *L. corniculatus* nodules. Deletion of the *T. tomentosa* promoter between nucleotides −199 and −169 removed a region required for detectable levels of GUS expression in developing *L. corniculatus* nodules.

The Consensus Motif AAGAG Is Required for High Levels of Nodule Expression

The *P. andersonii* deletion construct P-110, which contains both the AAAGAT and AAGAG motifs, is the minimal promoter able to confer histochemically detectable nodule expression in *L. corniculatus*. GUS expression was, however, lost (Table I) with the deletion from nucleotides −110 to −91 that removed the AAAGAT motif (Fig. 2), suggesting that this motif may play a critical role in nodule expression. *T. tomentosa* promoter constructs (T-137 or T-169) of equivalent or greater length to the P-110 construct contain both of these motifs and another upstream AAAGAT, but do not direct histochemically detectable GUS activity in nodules. The lower efficiency of these deleted *T. tomentosa* promoters may be either because the putative nodulin motifs are less well conserved or because of differences in other general promoter elements that determine absolute expression levels.

The importance of the AAAGAT and AAGAG motifs in the *P. andersonii* and *T. tomentosa* promoters in conferring nodule expression was examined by site-directed mutagenesis. Each putative nodulin box was mutated singly or in combination within the full-length promoter (Fig. 4). Mutation of the AAAGAT motif within the context of the whole promoter (construct P4) did not significantly alter GUS expression levels in nodules or roots (Fig. 5). On the other hand, changes to the AAGAG box (construct P5) reduced the level of nodule expression of GUS more than 9-fold (Fig. 5), suggesting that this motif is important for high-level expression of the *P. andersonii* promoter in *L. corniculatus*. The level of root expression was also significantly reduced (Fig. 5). The lack of effect of mutating the AAAGAT motif is supported by the observation that ex-

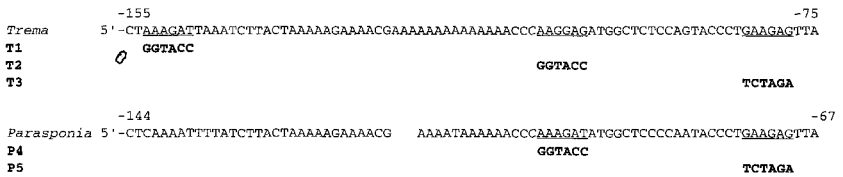

Figure 4. Site-directed mutagenesis of the putative nodulin motifs of the *P. andersonii* and *T. tomentosa* hemoglobin promoter regions. The two sequences are aligned and the sequence of the individual introduced mutations are shown in bold type. The sequences of the oligonucleotides used to introduce the mutations are given in "Materials and Methods" and putative nodulin consensus motifs are underlined. Numbering is relative to the transcription start site.

pression levels of the P4+5 double mutation are similar to that of the P5 mutation alone.

The AAAGAT and AAGAG motifs of the *T. tomentosa* promoter were tested, but the level of activity and low number of expressing transformants did not permit a statistically valid conclusion to be drawn, although the trends were similar to those seen with the *P. andersonii* transformants (not shown).

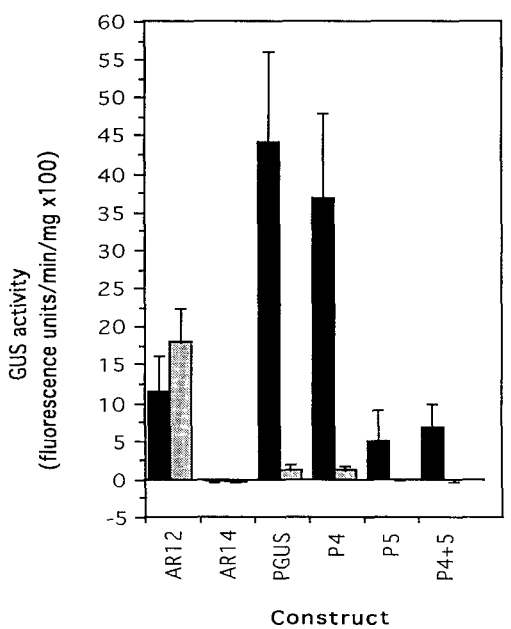

Figure 5. Quantitation of GUS activity of wild-type and mutant *P. andersonii* hemoglobin promoters in transgenic *L. corniculatus* nodules and roots. The full-length promoter was in vitro mutagenized as shown in Figure 4 to produce the nodulin motif mutant constructs either singly or in pairs. Mutant promoters were linked to the GUS reporter gene and transformed into *L. corniculatus* as hairy roots, as described in "Materials and Methods." PGUS represents roots transformed with the full-length promoter construct, whereas P4, P5, and P4+5 were roots transformed with the single- or double-mutant constructs, respectively. Values are the averages of GUS activities in either roots (stippled) or mature nodules (solid) and were determined for at least five independent transformants. SEs are indicated by the bars. AR12 or AR14 represents plants transformed with a constitutive CaMV 35S promoter GUS gene construct or 35S CAT gene, respectively.

Deletion of a 5′ Segment of the *P. andersonii* and *T. tomentosa* Promoters Alters the Spatial Pattern of Expression

Deletion of 5′ segments of both the *P. andersonii* and *T. tomentosa* promoters resulted in changes in the spatial distribution of reporter gene expression (Table I), with additional GUS staining observed in nonnodule tissues. In several transformants GUS activity was seen in cells of the root cap or in the root meristem initials (Fig. 3, K and L). GUS expression in root tips, when it occurred, was strongest in the most distal roots (Fig. 3L), including the primary root tip. Staining was progressively weaker in tips further up the root system. No expression was detectable in the root tips of the most proximal roots, and this did not correlate with root age because young laterals on the most proximal roots also did not stain. To investigate whether this expression pattern was related to the growth conditions in the culture pouches, where the distal roots were sometimes submerged in liquid medium, several plants were flooded for 24/48 h and analyzed for GUS expression. No discernible difference in the staining pattern was found between treated and untreated root systems. It appears that the root tip expression associated with these promoter deletions is determined by root system position rather than by environmental cues or root age.

In newly emerged lateral roots, reporter gene activity appeared to be strongest in the initial cells of the lateral root meristem (Fig. 3, M and N, construct P-371) or in the vascular tissue (not shown). Expression was initially restricted to a few cells that appeared as small blue spots on the primary root. While it is difficult to distinguish between lateral root and nodule meristems at these early stages, transgenic plants grown aseptically to prevent nodulation also showed GUS activity in identical lateral foci, confirming that the expression was in root primordia. This GUS activity was initially localized to dividing cells derived from the inner cortex and the pericycle (constructs T-278 in Fig. 3O), the site of lateral root initiation.

Histochemical analysis of the above-ground tissues of some promoter deletion transformants (Table I) localized GUS activity to primary and secondary pulvini (construct P-467 in Fig. 3P), the organs responsible for leaf movements. Sectioning showed expression to predominate in the flexor region of the pulvini (not shown). A similar pattern of expression has been seen for the full-length soybean Gln synthetase (GS15) promoter analyzed in trans-

genic *L. corniculatus* (Marsolier et al., 1993). GUS staining was not detected in all pulvini, but unlike the root tip expression, no consistent pattern of GUS activity relating to position or age of leaflets was found.

These ectopic expression patterns were seen in some plants containing *P. andersonii* promoter deletions from nucleotides −770 to −269 and for *T. tomentosa* deletions between nucleotides −651 and −244 (Table I).

The *P. andersonii* and *T. tomentosa* Promoters Are Expressed in the Indeterminate Nodules of White Clover

To determine whether the expression of the *P. andersonii* and *T. tomentosa* promoters was correlated with meristematic activity in the nodule, we examined these promoters in *T. repens* (white clover), in which meristematic activity continues throughout nodule development as a distinct zone at the distal end of the nodule. The promoters were generally expressed at a lower level in *T. repens*, but strong GUS staining was often observed in the tips of very young nodules (Fig. 3R); in older nodules expression was confined to the vascular traces (Fig. 3S) and the amyloplast-rich region (interzone II/III [Vasse et al., 1990]; Fig. 3T) just prior to the nitrogen fixation zone. Expression in the mature nodule decreased toward the proximal end and no staining was observed in the cells of the senescent zone (IV) (Fig. 3, S and T). This pattern of activity is qualitatively similar to that of the endogenous *T. repens* leghemoglobin genes (Scheres et al., 1990; de Billy et al., 1991) and of an *lbc3* GUS construct transformed into this same species (Lauridsen et al., 1993), although we have not determined the cell-type specificity of the expression in these white clover nodules. Very little, if any, GUS staining was detected in the meristematic zone (I) at the distal end of the older white clover nodule transformed with PGUS or TGUS constructs, so we must conclude that, although there is a general correlation with the expression of the *P. andersonii* and *T. tomentosa* promoters and zones of active cell division, expression is unlikely to be related directly to meristematic activity.

The deletion construct T-278 was also introduced into *T. repens*. It did not direct detectable GUS expression in nodules, but as in transgenic *L. corniculatus*, T-278 was active in the distal root caps or meristem initial cells (Fig. 3Q) and caps of emerging lateral roots.

DISCUSSION

The Cell-Type Expression in Transgenic *L. corniculatus* of the Hemoglobin Promoters from *P. andersonii* and *T. tomentosa* Suggests That Legumes May Regulate Symbiotic Hemoglobin Expression Differently than These Two Tree Species

We have shown that the patterns of GUS expression driven by the *P. andersonii* and *T. tomentosa* hemoglobin promoters in a transgenic legume, *L. corniculatus*, are similar but differ from that of the endogenous *L. corniculatus* leghemoglobins and from other leghemoglobin promoters analyzed in the same way. Expression from the *P. andersonii* promoter also differs from the distribution of hemoglobin protein in *P. andersonii* root nodules reported by Trinick et al. (1989) using immunolabeling with *P. andersonii* hemoglobin-specific antibodies. The antibodies detected high levels of hemoglobin in the cytoplasm of the bacteroid-containing cells and low levels in the uninfected interstitial cells. In the studies reported here the two nonlegume promoters were expressed in the central zone of the *L. corniculatus* nodules like the symbiotic leghemoglobins, but, unlike the leghemoglobins, they were expressed only at low levels in the bacteroid-containing cells (Nap and Bisseling, 1990). Both promoters showed greater relative activity in the uninfected interstitial cells of the central zone, the parenchyma of the inner cortex, and around the vascular bundle, and their expression is very similar to that reported for the *C. glauca* nonsymbiotic hemoglobin promoter in *L. corniculatus* nodules (Jacobsen-Lyon et al., 1995). This suggests that in *L. corniculatus* the hemoglobin promoters from *P. andersonii* and *T. tomentosa* are being regulated primarily as nonsymbiotic genes and that the symbiotic functions of the *P. andersonii* promoter are not being recognized.

It may be that having a single hemoglobin gene is unusual among nitrogen-fixing plants and that the *P. andersonii* gene has become specifically adapted to be expressed concurrently at different levels in different cell types in the *P. andersonii* nodules and roots to carry out its dual functions as a symbiotic and nonsymbiotic hemoglobin. It is likely that *L. corniculatus*, like soybean and other legumes, will have nonsymbiotic hemoglobins, as well as well-characterized symbiotic leghemoglobins (Andersson et al., 1996), and separate mechanisms to regulate the tissue and cellular distributions of both types. The imperfect regulation of the *P. andersonii* promoter in the legume presumably reflects generic differences and a failure to recognize some or all of the "symbiotic" regulatory sequences within the *P. andersonii* promoter that are normally recognized in a *P. andersonii* nodule.

Originating from a nonnodulating plant, the *T. tomentosa* hemoglobin is not expected to have the same dual roles as the *P. andersonii* gene, and its expression in transgenic *L. corniculatus* should be similar to other purely nonsymbiotic hemoglobins, such as that from *C. glauca*. Alternatively, *T. tomentosa* might once have been a nodulating species and its hemoglobin promoter might still have some relics of symbiotic expression, but the analysis presented here did not distinguish between the promoters from the two nonlegume species other than showing a quantitative difference in expression levels.

Critical Motifs for High-Level Expression Are Related to, but Different from, the Nodulin Motifs of the Leghemoglobins

The nonsymbiotic, nonlegume hemoglobin promoters contain conserved sequence motifs (Fig. 1) that resemble those implicated in the high-level, nodule-specific expression of the symbiotic hemoglobins. These motifs in the nonsymbiotic genes must only determine tissue- and not cell-type specificity because they cannot drive expression in the symbiotic cell types in transgenic *L. corniculatus*

nodules. Mutation of the AAGAG motif of the *P. andersonii* (and probably *T. tomentosa*) promoters revealed the importance of this positive *cis*-acting element for both nodule and root expression in transgenic *L. corniculatus*. The same motif is present in a comparable position in the *C. glauca* nonsymbiotic hemoglobin promoter (Fig. 1). In the symbiotic *C. glauca* hemoglobin gene and in the symbiotic leghemoglobin genes, a similar sequence is present but in a different position relative to the TATA box (Fig. 1). Ramlov et al. (1993) found that when the AAGAG motif in the *lbc3* leghemoglobin promoter was mutated, nodule expression was only halved, whereas we found that similar mutational changes in the AAGAG sequence in the *P. andersonii* gene essentially abolished the expression. This motif clearly plays a more critical role in the "nonsymbiotic" expression of the *P. andersonii* hemoglobin than it does in that of the symbiosis-specific hemoglobins of other species.

In the leghemoglobin genes, the CTCTT motif, the reverse complement of the AAGAG motif characterized here, has been identified as a sequence critical for nodule expression and forms one part of the OSE or NICE nodulin boxes (Stougaard et al., 1990; Szczyglowski et al., 1993). Perhaps the reverse orientation and change in position of this motif has been part of the evolutionary processes that gave rise to the more specific, cellular expression patterns of the symbiotic hemoglobin genes.

Whereas mutation of the AAAGAT motif, the other component of the leghemoglobin nodulin box, in the context of the full-length *P. andersonii* promoter suggested that it did not play a major role in nodule expression of the gene, the 5′ deletion experiments strongly implicated this sequence with the functioning of the promoter. Deletion of the promoter from nucleotide −110 to nucleotide −91 (spanning this motif) totally abolished histochemically detectable nodule expression of the reporter gene. Presumably, both the AAAGAT and AAGAG motifs are critical for nonsymbiotic hemoglobin expression, but the importance of the former is modulated by other upstream regulatory elements.

The *P. andersonii* and *T. tomentosa* Promoters Contain Similar Regulatory Elements Controlling Both Expression Level and Tissue Specificity

A series of 5′ deletions revealed the presence of a suite of regulatory sequences within the *P. andersonii* and *T. tomentosa* promoters. The approximate locations of these sequences, as defined by deletions and reporter gene activity detected histochemically, differs slightly between *P. andersonii* and *T. tomentosa*, but they show the same general order (Table I). The 5′ distal region of the promoter, upstream of −467 in *P. andersonii* and to −462 in *T. tomentosa*, showed evidence of a regulatory sequence(s) that repressed expression in nonnodule tissues. The promoters deleted at these end points showed activity in root tips, emerging lateral root primordia, and also the pulvini of the leaves (Table I). This upstream region in the *P. andersonii* promoter also contained a sequence(s) that had a major, positive effect on reporter gene expression in the more mature nitrogen-fixing nodules.

Regulatory sequences playing a minor role in mature nodule expression were also detected in the regions from −467 to −268 in the *P. andersonii* promoter. A further deletion, down to −235 (−199 in the *T. tomentosa* promoter), revealed a root tissue-specific positive regulatory element(s) distal to this end point. The deletion analysis indicated that the regulatory sequences controlling nonnodule expression and nodule-specific expression are likely to be different, or at most overlapping, because deletions down to −235 in *P. andersonii* and to −199 in *T. tomentosa* abolished all root and pulvini tissue activity but retained significant young nodule expression (Table I). Complete loss of nodule expression did not occur until after regions proximal to −110 (−199 in *T. tomentosa*) were removed (e.g. deletion constructs P-91 and T-169).

More detailed deletion and mutation analyses will be required to define the precise sequences of all of the regulatory elements inferred from these studies. There are clearly close correlations between the position and function of regulatory elements in the hemoglobin promoters from *P. andersonii* and *T. tomentosa* that are independent of the capacity of these species to be nodulated and fix nitrogen. These sequences probably represent the elements of the ancestral control machinery that are common to all nonsymbiotic hemoglobins and that may have been specifically refined in the more highly adapted species that contain both nonsymbiotic and symbiotic genes.

The Origins and Functions of Plant Hemoglobins

The widespread occurrence of the nonsymbiotic hemoglobins has suggested to us that they are the direct progenitors of the symbiotic genes (Appleby et al., 1990). However, if this were true, the differences in the regulatory machinery controlling the cell specificity of the nonsymbiotic hemoglobin genes would have to have been changed dramatically during the evolution of nodulation. In transgenic *L. corniculatus* the *cis*-regulatory elements in the promoters of the *P. andersonii*, *T. tomentosa*, and *C. glauca* nonsymbiotic hemoglobin genes do not confer the appropriate cell specificity for a role in symbiotic nitrogen fixation, and to evolve into a symbiotic hemoglobin, a nonsymbiotic gene would have to considerably elevate its expression in bacteroid-containing cells while suppressing expression in nonsymbiotic tissues. Some of the basic elements for symbiotic expression are presumably present in the two promoters we have examined. There are upstream regions that supress nonnodule expression of the *P. andersonii* promoter and there are distinct sequence similarities between the important nodulin motifs of the leghemoglobins and critical sequences in the *P. andersonii* promoter. The control of cell specificity within the nodule, however, must have had different origins in the *P. andersonii* gene to the leghemoglobins and symbiotic hemoglobin from *C. glauca*, because these latter symbiotic genes are all faithfully expressed in the correct cell type in transgenic *L. corniculatus*. The similarities and differences of the regulatory motifs between the *P. andersonii* and *T. tomentosa* promoters and those in the legumes clearly suggest a common

evolutionary origin for both types of hemoglobin, but it may be that the nonsymbiotic forms are not direct progenitors of the symbiotic forms but have a common progenitor that at some point diverged significantly to result in totally different patterns of expression, presumably correlated with the evolution of their different physiological functions.

The activity of the full-length *P. andersonii* and *T. tomentosa* hemoglobin promoters in *L. corniculatus* and some of the deletion constructs was generally seen in tissues undergoing rapid cell division (e.g. the parenchyma of the developing nodule or root primordia). If this represents predominantly nonsymbiotic expression, it may be a clue to the function of these genes in nonlegume species. Dividing tissues might be expected to have an increased need for a stable oxygen supply, and the oxygen-binding properties of hemoglobin may ensure that respiration was not limiting in such cells in the presence of high localized concentrations of the hemoglobin protein. Although staining with the GUS reporter gene appeared particularly high in the cells of the nodule meristem (Fig. 3I), in a different species with indeterminate nodules there was little GUS staining of the persistent meristem of mature nodules (Fig. 3, S and T). This suggests that, although *P. andersonii* and *T. tomentosa* hemoglobin promoter function do correlate with areas of active cell division, they are not exclusively associated with meristematic activity but may be influenced by other physiological factors such as hormone levels or oxygen tensions within particular tissues. The more intense staining observed in *L. corniculatus* nodule meristems could perhaps simply reflect higher cell or cytoplasmic densities in the meristematic zone of the determinate nodules.

The true physiological determinants of the cellular specificity and the functions of the nonsymbiotic hemoglobins in plants remain to be elucidated. This study highlights the precision with which genes are regulated in particular cell types and the ways different plants have evolved to refine the controls over individual and highly regulated genes.

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